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Alcohol Contributes to Attraction of *Heliothis (= Chloridea) virescens* Males to Females

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Abstract

Female-emitted volatile sex pheromones in most moths are composed of biosynthetically related blends of fatty acid derivatives, such as aldehydes, acetate esters and alcohols. In many moths, as in the noctuid *Heliothis (Chloridea) virescens*, the pheromone gland contains alcohols (e.g., (*Z*)-11-hexadecen-1-ol, hereafter *Z*11–16:OH) that may serve dual functions as pheromone components as well as precursors of other pheromone components. The relative importance of *Z*11–16:OH to male attraction in *H. virescens* has been controversial. It occurs in the pheromone gland in relatively large amounts, but several studies could neither detect *Z*11–16:OH in gland emissions nor attribute any conspecific behavioral function to it in flight- tunnel assays. Trapping assays in the field, however, have more consistently documented that the addition of *Z*11–16:OH increased trap catch. Using a short section of thick film megabore column, in combination with derivatization and GC-CI-SIM-MS, we determined that *Z*11–16:OH is emitted from the sex pheromone gland during calling. Field trapping studies demonstrated that trap catch increased when *Z*11–16:OH was added to a 2-component minimal blend and to a 6-component blend. Behavioral observations in the field confirmed that more males responded to a pheromone blend that contained a low blend ratio of *Z*11–16:OH, but ≥5% *Z*11–16:OH depressed both male behavior and trap catch. We conclude that *Z*11–16:OH should be considered a component of the sex pheromone of *H. virescens* females.

Keywords *Heliothis virescens* · *Chloridea virescens* · Sex pheromone · Volatile collection · (*Z*)-11-Hexadecen-1-ol · Rubber septa · Behavior

Introduction

Sex pheromone gland compounds have been identified from gland extractions in many moth species (El-Sayed 2018), but it is often unclear which of these actually function as pheromone components. That is, do they play a role in attracting conspecific males? In addition to pheromone components, the pheromone gland may contain precursors of pheromones, compounds that antagonize the attraction of heterospecifics, or related metabolic products that play no clear role in sexual attraction (Allison and Cardé 2016a; Jurenka et al. 2016). "Minimal" blends generally consist of the major sex pheromone component, which is often, but not always, the most abundant compound in the pheromone gland, plus one or

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more critical secondary components. The secondary component(s) are often present in minor amounts, but without it conspecific males are not attracted (Linn and Roelofs 1989).

Evaluating which compounds are important in the sexual communication of a species is not trivial, because in general there is redundancy in moth sex pheromone compositions, and it is difficult to detect the behavioral effects of relatively minor components. Complete blends that include all the compounds known to function in sexual attraction are more attractive than minimal blends (Vetter and Baker 1983; Groot et al. 2007), although the latter are often formulated for commercial applications (Witzgall et al. 2010; Suckling 2016).

To understand the evolution of sexual communication, it is critical to assess the behavioral and biochemical relevance of all compounds contained in and released from the pheromone gland. In general, compounds that do not significantly increase the attraction of conspecific males may nonetheless be under selection, if they serve as pheromone precursors and/or inhibit the attraction of heterospecific males (Allison and Cardé 2016b; Jurenka et al. 2016). The contribution of each pheromone gland component to attracting conspecific males can be tested by either adding each compound separately to the minimal blend or subtracting it from a complete blend. However, small but significant effects are often difficult to quantify in complex multi-component blends.

The female-emitted sex pheromone of the noctuid *Heliothis (Chloridea) virescens* was identified as consisting of (*Z*)-11-hexadecenal (*Z*11–16:Ald) as the major sex pheromone component and (*Z*)-9-tetradecenal (*Z*9–14:Ald) as the critical secondary sex pheromone component (Roelofs et al. 1974; Tumlinson et al. 1975). This binary minimal blend can elicit attraction of males in the field and in flight tunnel assays. Subsequently, other compounds were identified in the pheromone gland: tetradecanal (14:Ald), hexadecanal (16:Ald), (*Z*)-7-hexadecenal (*Z*7–16:Ald), (*Z*)-9-hexadecenal (*Z*9–16:Ald), tetradecen-1-ol (14:OH), hexadecen-1-ol (16:OH), (*Z*)-9-hexadecen-1-ol (*Z*9–14:OH), and (*Z*)-11-hexadecen-1-ol (*Z*11–16:OH) (Klun et al. 1979; Klun et al. 1980; Teal et al. 1986). The relative amounts of these compounds and their contribution to male attraction have been controversial, however. For example, Klun et al. (1980) demonstrated in laboratory assays that the addition of other pheromone gland components did not elicit greater responses than a mix of the two essential components (*Z*11–16:Ald and *Z*9–14:Ald); however, a 7-component mix of *Z*11–16:Ald, *Z*9–16:Ald, *Z*7–16:Ald, 16:Ald, *Z*11–16:OH, *Z*9–14:Ald, and 14:Ald was 5–6-fold more attractive to males in field trapping assays. Nevertheless, the role of each individual component in the blend remained uncertain because emission from the gland was not determined and only some combinations of these compounds were tested. Pope et al. (1982) demonstrated that in addition to *Z*11–16:Ald and *Z*9–14:Ald, two saturated aldehydes (16:Ald and 14:Ald) were also emitted from the pheromone gland, and the unsaturated aldehydes (*Z*7- and *Z*9–16:Ald) were emitted only in small amounts and inconsistently. However, even the emitted compounds were considered nonessential for male attraction since their omission did not significantly affect male upwind flight in a flight tunnel (Vetter and Baker 1983). In some assays, 16:Ald appeared to increase close-range sexual behaviors, and Vetter and Baker (1983) therefore considered it a pheromone component. These findings were confirmed in an integrated study of gland extracts, gland volatiles, volatile emission from calling females, and behavior, that concluded that all six of the 14- and 16-carbon aldehydes were emitted and served as pheromone components (Teal et al. 1986).

The relative importance of the alcohol, *Z*11–16:OH, in *H. virescens* has been particularly controversial. It occurs in the pheromone gland in relatively large amounts, but this has been attributed to its role as a precursor of the major sex pheromone component, *Z*11–16:Ald (Teal and Tumlinson 1986; Morse and Meighen 1987; Choi et al. 2005). Various studies could neither detect *Z*11–16:OH in pheromone gland emissions (Pope et al. 1982; Teal et al. 1986; Heath et al. 1991) nor attribute any conspecific behavioral function to it in flight tunnel assays (Vetter and Baker 1983; Teal et al. 1986). Trapping assays in the field, however, have more consistently demonstrated that the addition of *Z*11–16:OH significantly increased trap catch over the 2-component minimal blend (Hartstack et al. 1980). For example, the 7-component-Klun et al. (1980) blend, which included *Z*11–16:OH, was superior to the 2-component blend and comparable to three virgin pheromone-emitting females (Ramaswamy et al. 1985). In addition, *Z*11–16:OH improved trap catch of both the 2- and 6-component aldehyde blends in Shaver et al. (1989). Moreover, it became apparent that *Z*11–16:OH increased trap catch only when formulated at relatively low blend ratios (~1%), whereas higher amounts (>5%) significantly reduced trap catch (Ramaswamy et al. 1985; Shaver et al. 1989). Yet, in a recent review of heliothine sex pheromones, Hillier and Baker (2016) surprisingly consider only *Z*11–16:Ald and *Z*9–14:Ald as required components for *H. virescens* male attraction, with *Z*9–14:Ald at a remarkably broad range of 5–50% relative to *Z*11–16:Ald (set at 100%), based on Vickers et al. (1991). Despite the field evidence to the contrary, Hillier and Baker (2016) consider *Z*11–16:OH as strictly a heterospecific pheromone component that reduces attraction of *H. virescens* males.

The fickleness of these assays is demonstrated by the findings that addition of *Z*11–16:OH to a 2–4 component "minimal blend" significantly increased trap catch, but its removal from the 7-component blend had minimal effects (Ramaswamy et al. 1985). Other confounding factors in these studies include: (1) *Z*11–16:OH is relatively less volatile than homologous aldehydes, making it more difficult to detect in headspace collections; (2) At low concentrations, alcohols—

including *Z*11–16:OH—are more difficult to analyze by GC than related aldehydes, and readily adsorb to glass surfaces; (3) Reagent grade compounds, such as *Z*11–16:Ald, may contain *Z*11–16:OH as a contaminant; (4) *Z*11–16:OH clearly inhibits male attraction at high concentrations relative to *Z*11–16:Ald, but the shape of the dose-response curve is unknown; and (5) Different dispensers and doses were used by various researchers, while it is known that high doses of essential components can obscure the effects of minor components tested in blends at lower doses (Linn et al. 1987).

To evaluate the relative contribution of *Z*11–16:OH to male attraction as a component of the sex pheromone blend of *H. virescens*, we determined a) whether it is actually emitted from the female sex pheromone gland during calling, and b) whether trap catch is affected when it is added to a minimal blend or subtracted from a more complex blend.

Methods and Materials

Insects The YDK strain of *H. virescens* originated from eggs that were collected from tobacco plants in Yadkin County, NC, in 1988. Pupae were sexed, and female pupae were transferred to a reverse L:D (16:8) room (L off 08:00 h, ambient RH 35–80%) and checked daily for emergence. Newly emerged females were placed in 0.5 l buckets, provided 10% sucrose in water, and maintained in the same room as the pupae. For the volatile collections and subsequent gland extractions, 2–4 day old virgin females were used.

Volatile Collection and Gland Extraction To maximize interaction of emitted compounds with the adsorbent and minimize solvent use for pheromone elution, we developed a new collection apparatus based on previous work (Nojima et al. 2008; Nojima et al. 2011; Kuenen and Hicks 2015). The collection procedure was developed and validated with authentic aldehydes, acetate esters and alcohols, and with *Heliothis subflexa*, which emits a relatively large amount of *Z*11–16:OH (Nojima et al. 2018). The adsorbent trap consisted of a 20 cm section of thick film DB-1 column (0.53 mm ID, 5 μm film thickness) connected to a vacuum pump that delivered a flow rate of 10 ml/min. A silanized glass funnel, made from a Pasteur pipet, was attached to the upwind end of the column. The funnel was carefully positioned so the ovipositor of a calling female was 1–3 mm below the lip of the funnel, and volatiles were collected from the ambient air. The vacuum pump was turned off when the gland was retracted, and turned back on when calling resumed, for 30 min of total calling duration. Control collections of ambient air were subtracted from the collections from calling females.

The 20 cm DB-1 column was eluted with 10 μl hexane containing 20 ng internal standard (pentadecyl acetate, 15:OAc), followed by two 10-μl elutions with clean hexane. The eluted hexane was collected in a 300 μl conical glass insert (Agilent) which was stored in a 1.5 mL autoinjection vial in a -30 °C freezer until use.

After collecting volatiles from one or more females, the pheromone gland of each female was dissected and extracted for 15 min in 50 μl hexane containing 100 ng 15:OAc as internal standard. Gland extracts were derivatized and processed by GC-MS.

Derivatization and GC-MS Analysis Each sample was reduced to 10 μl under a gentle stream of high purity N_2. To derivatize the alcohols we added 3 μl of 1% BSTFA (*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide) with 10% TMCS (trimethylchlorosilane) (Pierce, Rockford, IL, USA) in hexane. The sample was carefully mixed and briefly centrifuged to draw all the solution to the bottom of the vial. The reaction mixture was then incubated for 30 min at room temperature, reduced to 2–3 μl with N_2, the inside wall of the vial was washed with 5 μl hexane, reduced to 2–3 μl, and the total reaction mixture was injected into the GC-MS. This procedure adds a trimethylsilyl (TMS) functional group to the compound.

We used a 6890 GC (Agilent) coupled to a 5975 MSD (Agilent) and equipped with a DB-5 ms column (30 m × 0.25 mm × 0.25 μm, Agilent) with a 2-m retention gap as a guard column. Injector temperature was 270 °C and samples were introduced in pulsed splitless mode (20 psi for 2 min, then 9.4 psi). Helium was the carrier gas at a constant flow of 1.2 ml/min. The oven was programmed from 50 °C (2 min hold) to 210 °C at 4 °C/min and then to 260 °C at 20 °C/min (10 min hold). The MS was operated in chemical ionization (CI) mode with methane as reagent gas. We monitored the following ions by SIM (*m*/*z*): *Z*9–14:Ald (209, 211, 239); 14:Ald (211, 213, 241); *Z*7–16:Ald (237, 239, 267); *Z*9–16:Ald (237, 239, 267); *Z*11–16:Ald (237, 239, 267); 16:Ald (239, 241, 269); 15:OAc (internal standard, 269, 271, 311); *Z*9–16:OH(TMS) (297, 311); and *Z*11–16:OH(TMS) (297, 311).

Field Trapping Experiments To determine the effect of *Z*11–16:OH on *H. virescens* male attraction, we formulated sex pheromone lures for field experiments in 2005, 2007, 2013 and 2014. Treatment blends were made relative to the major component, *Z*11–16:Ald (see Table 1), which was loaded into rubber septa at 300 μg (2005, 2007) or 100 μg (2013 and 2014). Pheromone components were obtained from PHEROBANK (Wageningen, The Netherlands), Shin Etsu (Tokyo, Japan) and Bedoukian (Danbury, CT, USA) with >95% purity, and dissolved in hexane. In 2013 and 2014, *Z*11–16:Ald was purified on a silica gel column to ensure that alcohol contaminants were removed. Butylated hydroxytoluene (BHT) was added at 1% (*w*/*v*) to solutions in 2005 and 2007 but not in 2013 and 2014, and ratios were confirmed by

Table 1 Pheromone lures used to determine whether *Z*11–16:OH contributes to attraction of *H. virescens* males in the field

Component	Dates	Jul 7–29 & Aug 10–Sept 6 2005; Aug 4–13 2007	Sept 8–13 2013; Aug 16–Sept 13 2014	Sept 12–22 2005 (NC); Aug 4–9 (TX)
	Location	NC	NC	NC, TX
	Lure [a]	Minimal [b]	Intermediate [c]	Complete [d]
*Z*11–16:Ald		300 μg (100%)	100 μg (100%)	300 μg (100%)
*Z*9–14:Ald		15 μg (5%)	5 μg (5%)	15 μg (5%)
14:Ald			5 μg (5%)	15 μg (5%)
16:Ald			10 μg (10%)	30 μg (10%)
*Z*7–16:Ald				6 μg (2%)
*Z*9–16:Ald				6 μg (2%)
*Z*11–16:OH		0, 3 μg (0%, 1%)	0, 0.5, 1, 5, 10 μg (or %)	0, 3 μg (0%, 1%)

[a] Lure was a rubber septum; Percentages are relative to *Z*11–16:Ald

[b] Addition of *Z*11–16:OH to a 2-components minimal aldehyde blend

[c] Addition of *Z*11–16:OH to a 4-components intermediate aldehyde blend

[d] Addition of *Z*11–16:OH to a 6-components complete aldehyde blend

GC-FID on a DB-WAXetr (extended temperature range) column (30 m × 0.25 mm × 0.25 μm). Red rubber septa (#1780 J07, Thomas Scientific, Philadelphia, PA, USA) were first ultra-sonicated for 6 h in hexane, then extracted ×3 with hexane, and air-dried in a fume hood for at least 48 h before loading. Each septum was first loaded with 100 μl of one of the treatment solutions, air-dried, then 100 μl of hexane was added to the septum well, air-dried again, and stored in glass vials at −30 °C until used. Red rubber septa had been used previously in field studies with *H. subflexa* (Heath et al. 1990) and *H. virescens* (Ramaswamy et al. 1985), including by us (Groot et al. 2007; Groot et al. 2009; Groot et al. 2010; Groot et al. 2014).

The effect of *Z*11–16:OH was tested by adding it to a 2-component minimal blend (Table 1) in Clayton, NC (35° 39′ 58″ N, 78° 30′ 36″ W) twice in 2005 (July 7 to 29: 3 trap pairs over 8 trapping sessions = 24 trapping events; August 10 to September 6: 3 pairs of traps over 8 trapping sessions = 24 trapping events per treatment) and once in 2007 (August 4 to 13; 3 pairs of traps rotated over 5 trapping sessions = 15 trapping events per treatment). In 2005 we also assessed the effect of adding *Z*11–16:OH to a 6-component blend (Table 1) in NC and TX (College Station, Texas: 30° 38′ 22” N, 96° 21′ 39” W). The NC experiments were conducted September 12 to 22 (4 pairs of traps rotated over 5 trapping sessions = 20 trapping events per treatment), and in TX (August 4 to 9; 4 pairs of traps rotated over 4 trapping sessions = 16 trapping events per treatment). In 2013 (September 8 to 13) in NC we added 0 to 10% *Z*11–16:OH to a 4-component intermediate blend (Table 1) (5 treatment blocks rotated over 5 days = 25 trapping events per blend ratio). We used locally-constructed modified metal screen Hartstack *Heliothis* traps. Traps were positioned at least 15 m apart in a soybean field. The septa were hung on a metal clip positioned in the center of the trap opening. All septa were replaced at the same time, every 1–2 wk., and treatments were rotated every 1–3 days, such that each trap position received both treatments, to account for location effects. Trap catches were brought back to the lab to identify the captured moths and count the number of *H. virescens* males.

Behavioral Observations in the Field Since trap catches do not account for males that approach the lure but do not enter the trap, we conducted night-time observations in 2014 (August 16 to September 13) using treatments as in 2013 (Table 1). Observations were conducted in a soybean field in Clayton NC from 22:00 h until 03:00 h, where ‘approach’, ‘within 50 cm’, and ‘touch’ the lure were noted for every *H. virescens* male that was observed. In this experiment, a single rubber septum lure was placed next to a pinned dead female placed on a soybean leaf in a soybean field and observed for 15 min. The lure (dead female and septum) and leaf were removed and 5 min later another septum and pinned female were placed 10 m away and observed. This procedure was repeated until all five treatments were completed (see Table 1), and the experiment was replicated 5 times. All observations were made with the aid of a red LED headlamp.

Statistical Analysis Pheromone gland contents and release rates of volatiles were compared with the Wilcoxon test ($\alpha = 0.05$). For the field tests with the minimal blend conducted in 2005 and 2007 in NC, and for the field tests with the complete blend conducted in 2005 in NC and TX, treatments with 0 and 1% *Z*11–16:OH were compared in JMP Pro 13.1.0 (JMP Pro 2016) using a paired Student’s *t*-test with trap location defining paired treatments.

For the dose-response experiment in 2013, we conducted two types of analyses. One was an ANOVA followed by Tukey-Kramer honest significant difference (HSD) multiple comparison ($\alpha = 0.05$). For analysis of percentages, data were

transformed as arcsin(sqrt(x + 0.5)). A generalized additive model (GAM), useful for checking the effect of a continuous independent variable without *a priori* assumptions about the particular shape of the function, was also used. The GAM assumed a Poisson error distribution (typical for count data) and used generalized cross validation to fit smoothing parameters to the relationship between trap catch and percent *Z*11–16:OH in the blend. The *mgcv* package (Wood 2011) in R v. 3.0.2 (R Core Team 2013) was used for this analysis with base dimension set to k = 4.

For the 2014 field observations of the effects of percentage representation of *Z*11–16:OH added to a 4-component blend, lures were prepared as in 2013. We conducted two types of analyses. One was an ANOVA followed by Tukey-Kramer HSD multiple comparison ($\alpha = 0.05$). We also coded the data such that approach = 1, within 50 cm = 2, and touch = 3, and used survival analysis to assess if there were statistical differences between Kaplan-Meier curves for each treatment. Using R (version 3.4.4), first a Cox proportional hazards model (R package *survival*) was fitted and subsequently differences between Kaplan-Meier curves were assessed using a Tukey test (R package *multcomp*). In this analysis the sequential steps – approach, within 50 cm and touching the odor source – are assumed to be time steps while the discontinuation of this sequence is analogous to a death event in regular survival analysis. Because moths can only discontinue the behavioral sequence after approaching an odor source, we omitted septa that did not attract any moths from this analysis (2 at 0%, 1 at 1%, 2 at 5% and 3 at 10% *Z*11–16:OH). Differences between Kaplan-Meier curves therefore indicate differences only in the close range (within 50 cm, or touch) continuation or abortion of the approach.

Results

Volatile Emissions and Relationship to Pheromone Gland Content First, we validated the use of a 20-cm section of thick-film DB-1 column as a volatiles trap by applying compounds on a glass bead (representing the pheromone gland) and collecting volatiles for 30 min onto four 20-cm sections of column connected in series. All the volatiles were found in the first 20-cm section (Nojima et al. 2018). Next, we confirmed that air flow over the glass bead did not oxidize the aldehydes by collecting volatiles from standards spiked on a glass bead using helium. We found no differences between air collections and helium collections (Nojima et al. 2018), indicating that these compounds remained intact. We also compared air flows of 5, 10 and 15 ml/min. Although 15 ml/min was slightly more effective with standard chemicals, we settled on 10 ml/min because higher flow rates generated high linear velocities that appeared to disturb the female's calling (Nojima et al. 2018).

We collected volatile emissions from four calling *H. virescens* females and then extracted their pheromone gland contents. The following compounds were readily detected by GC-MS: *Z*9–14:Ald, 14:Ald, *Z*7 + *Z*9–16:Ald, *Z*11–16:Ald, 16:Ald, and *Z*11–16:OH(TMS) (Fig. 1). *Z*9–16:OH(TMS) was found in the gland in small amounts, but its recovery from volatile collections was inconsistent. We did not find any of the three *H. subflexa* acetate esters, *Z*7–16:OAc, *Z*9–16:OAc and *Z*11–16:OAc, in *H. virescens* glands or volatile collections. Most importantly, *Z*11–16:OH was detected as its TMS-derivative in all collections, but in very small amounts, ranging from 0.03 to 0.06 ng per 30 min of volatile collection (Fig. 1). For all the aldehydes, the amount of each compound emitted in 30 min of calling was similar to the mass of the respective compound extracted from the pheromone gland. Overall, even with only four females, we found a positive correlation (Spearman's rho, ρ) between the amount of each aldehyde extracted from the pheromone gland and its emission prior to extraction, but only correlations for *Z*9–14:Ald and 16:Ald were significant: *Z*9–14:Ald, $\rho = 0.956$, $P <$

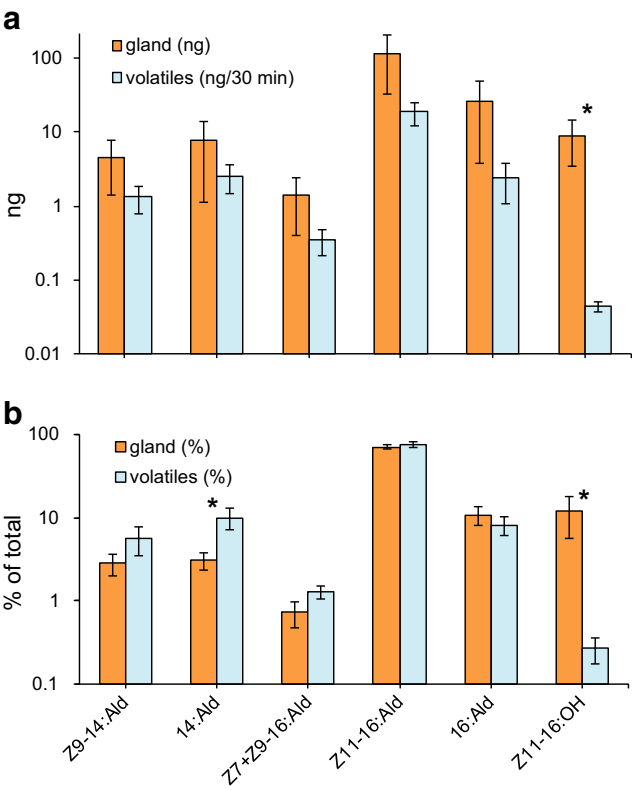

Fig. 1 Relationship of pheromone gland extracts and volatiles collected from four calling *H. virescens* females. Volatiles were collected for 30 min and the gland was subsequently extracted. All samples were derivatized to maximize detection of *Z*11–16:OH and processed by GC-MS. **A**. Mass (ng) of compounds extracted from the gland (orange) and trapped from collections of volatiles (blue). **B**. Relative content (%) of each compound in the pheromone gland (orange) and collections of volatiles (blue), calculated relative to the total amount of all components. Note logarithmic scale of the y-axes. Variation is represented by SEM. * $P < 0.05$ (Wilcoxon test)

0.0001; 14:Ald, ρ = 0.800, $P = 0.200$; *Z*7 + *Z*9–16:Ald, ρ = 0.400, $P = 0.600$; *Z*11–16:Ald, ρ = 0.400, $P = 0.600$; and 16:Ald, ρ = 0.968, $P < 0.0001$. For *Z*11–16:OH, however, significantly less was emitted than present in the gland, and we found a negative correlation with ρ = −0.807, $P < 0.001$, possibly highlighting its dual role as an emitted pheromone component and as precursor of the major pheromone component *Z*11–16:Ald.

Z11–16:OH Is a Pheromone Component: Field Trapping Experiments In experiments conducted July and August 2005 and August 2007 in NC, we added 3 μg (1%) *Z*11–16:OH to a 2-component minimal blend containing 300 μg *Z*11–16:Ald and 15 μg *Z*9–14:Ald. In the first experiment in July 2005, traps baited with 1% *Z*11–16:OH caught 38% more males than traps without *Z*11–16:OH, but the two treatments were not significantly different (Fig. 2). However, in the next two experiments (August–September 2005 and August 2007), and when all three experiments were combined, significantly more males were caught when 1% *Z*11–16:OH was added to the 2-component blend (Fig. 2).

In two independent experiments with a 6-component 'complete' blend, conducted in NC (September 2005) and TX (August 2005), the addition of 1% *Z*11–16:OH significantly increased trap catch by 68 and 322%, respectively (Fig. 3).

In September 2013 we used a 4-component intermediate aldehyde blend (*Z*11–16:Ald, *Z*9–14:Ald, 14:Ald and 16:Ald), with rubber septa loaded with only 33% of the amount loaded in prior experiments (i.e., only 100 μg *Z*11–16:Ald). The addition of 0.5 and 1% *Z*11–16:OH resulted in corresponding increases in trap catch, with 1% *Z*11–16:OH significantly increasing trap catch by 94% relative to the 4-component blend alone

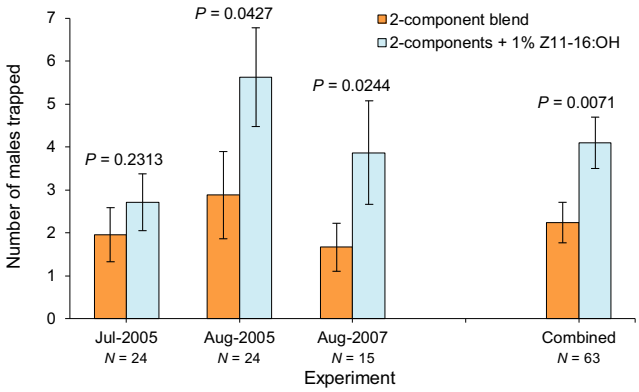

Fig. 2 Trap catches of *H. virescens* males in North Carolina in 2005 and 2007, using lures with the 2-component minimal blend (300 μg *Z*11–16:Ald and 15 μg *Z*9–14:Ald) to which 0% or 1% (3 μg) *Z*11–16:OH was added. Mean (± SEM) number of males caught in each experiment. Number of trapping events per treatment shown under each bar label. "Combined" represents the combined data from all experiments in 2005 and 2007. *P*-values based on paired Student's *t*-tests by trap location

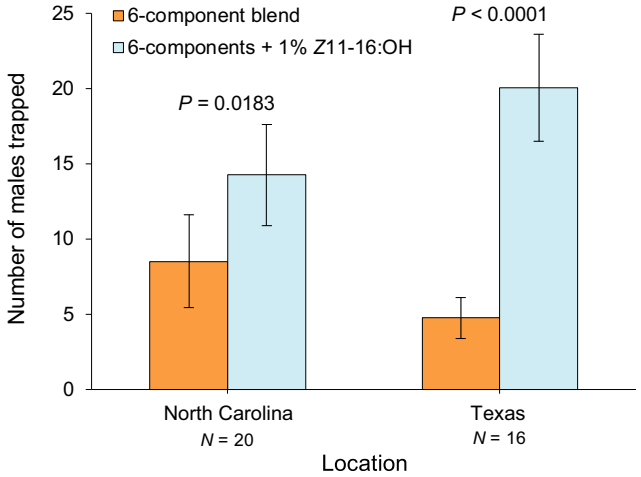

Fig. 3 Trap catches of *H. virescens* males in North Carolina and Texas in 2005, using lures with a 6-component blend (300 μg *Z*11–16:Ald, *Z*9–14:Ald, 14:Ald, 16:Ald, *Z*7–16:Ald and *Z*9–16:Ald) to which 0% or 1% *Z*11–16:OH was added. Mean (± SEM) number of males caught in each experiment. Number of trapping events per treatment shown under each bar label. *P*-values based on Student's paired *t*-tests by trap location

(Fig. 4a). Further increases in *Z*11–16:OH to 5 and 10% significantly depressed the number of males caught in traps. This "hump shaped" pattern with a peak at 1% *Z*11–16:OH was also evident when data were normalized to percentages (Fig. 4b). GAM analysis revealed a significant smoothing parameter ($\chi^2 = 86.4$, edf = 2.965, rdf = 2.999, $P < 0.0001$) suggesting a non-linear relationship and a predicted optimal level of *Z*11–16:OH in the 5-component blend between 1 and 5% for red-rubber septa (Fig. 4c).

Z11–16:OH Is a Pheromone Component: Behavioral Observations in the Field Using lures identical to the 2013 lures we conducted nightly behavioral observations in a soybean field in August–September 2014. The pattern was remarkably similar to the trapping results, with significantly more *H. virescens* males approaching, reaching and contacting lures that contained 1% *Z*11–16:OH than lures that did not contain this alcohol, while all other lures (with 0.5%, 5 and 10% *Z*11–16:OH) were not significantly different from 0% *Z*11–16:OH (Fig. 5a-c). Kaplan-Meier analysis confirmed that only the lures containing 1% *Z*11–16:OH caused significantly more males to approach the lure than those that did not contain *Z*11–16:OH (Z = −3.148, $P = 0.0016$) (Fig. 5d).

Discussion

Our main findings are that (a) *Z*11–16:OH is emitted from the pheromone gland of *H. virescens* females, albeit at extremely low rates that would be particularly difficult to detect without derivatization and silanization

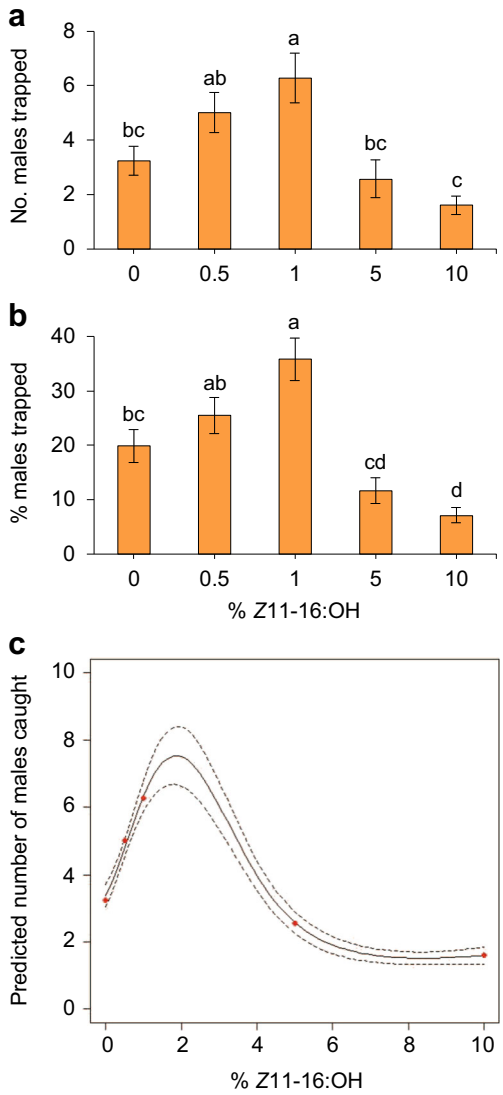

Fig. 4 Trap catches in an experiment in 2013 in North Carolina using a 4-component blend (100 μg *Z*11–16:Ald, *Z*9–14:Ald, 14:Ald and 16:Ald) to which various percentages of *Z*11–16:OH were added ($N = 25$ trapping events per treatment). **a**. Number of males caught in each treatment. **B**. Percentage of total males caught in each treatment. Different letters above the bars indicate significant differences (ANOVA, Tukey-Kramer HSD multiple comparison test, $P < 0.05$). In **b**, % males trapped was transformed as arcsin(sqrt(x + 0.5)). **c**. Generalized additive model (base dimension, k = 4) for the relationship between trap catch and percent *Z*11–16:OH in the blend. Solid circles indicate actual means and the dotted lines are ±1 SE Poisson errors on the model fit. Approximate significance of the smooth terms: estimated df = 2.965, ref.df = 2.999, Chi square = 86.44, $P < 0.0001$

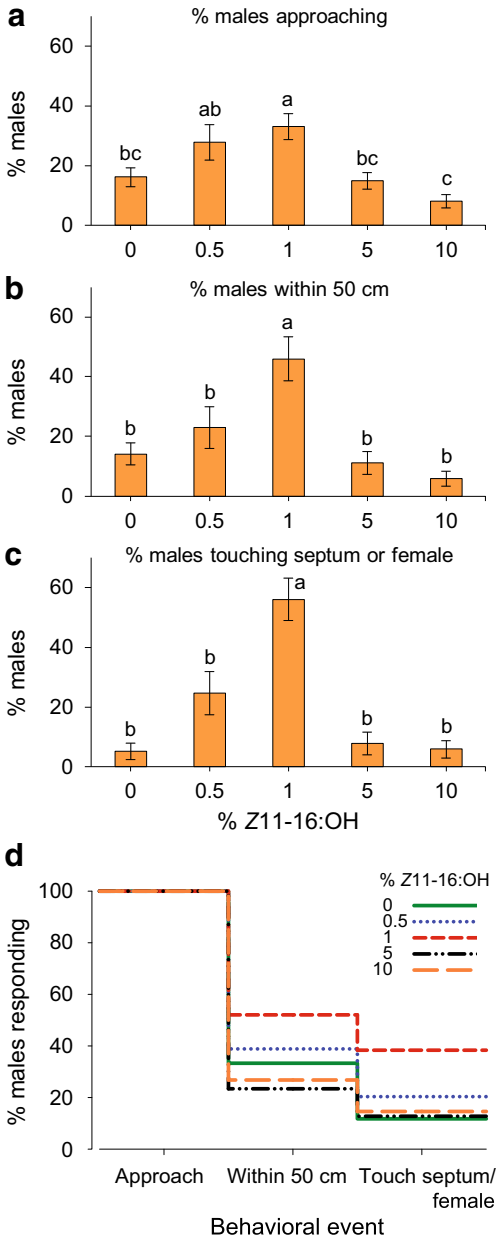

Fig. 5 Responses of male *H. virescens* in the field to septa loaded with a 6-component blend (100 μg *Z*11–16:Ald, *Z*9–14:Ald, 14:Ald, 16:Ald, *Z*7–16:Ald and *Z*9–16:Ald) and various percentages of *Z*11–16:OH ($N = 15$ replicates of each blend). Each septum was placed next to a dead pinned *H. virescens* female on a soybean leaf. **A**. Percentage of total males observed in the field that approached each septum. **B**. Percentage of total males observed in the field that reached within 50 cm of the septum. **C**. Percentage of total males observed in the field that touched the septum/female. **D**. Kaplan-Meier curves showing the percentage of males that were attracted reaching each of the following two stages (Wald test = 23.47, df = 4, $P = 0.0001$). The lures to which 1% *Z*11–16:OH was added performed significantly better than lures that contained 0% (Z = −e3.939, $P < 0.001$), 5% (Z = e4.143, $P < 0.001$) and 10% *Z*11–16:OH (Z = 3.544, $P = 0.004$). There were no significant differences in the close-range behaviors between any other treatments. In **A-C**, different letters above the bars indicate significant differences (ANOVA, Tukey-Kramer HSD multiple comparison test, $P < 0.05$). Field observations were conducted in 2014, using the same blends as in 2013 (see Table 1)

of glassware, and b) the addition of 1% *Z*11–16:OH to a 2-component minimal blend, a 4-component intermediate blend, or a 6-component complete blend significantly increased the attraction of *H. virescens* males. These results support the assertion that *Z*11–16:OH should be considered a component of the sex pheromone of *H. virescens* females, not just a compound present in their pheromone gland.

The composition of the *H. virescens* sex pheromone blend has been contentious since its first characterization as a 2-component aldehyde blend—*Z*11–16:Ald and *Z*9–14:Ald (Roelofs et al. 1974; Tumlinson et al. 1975). Tumlinson et al. (1975) suspected a more complex blend, and over the following decade, eight more compounds were identified in the pheromone gland of *H. virescens*: 14:Ald, 16:Ald, *Z*7–16:Ald, *Z*9–16:Ald, 14:OH, 16:OH, *Z*9–14:OH and *Z*11–16:OH (Klun et al. 1979; Klun et al. 1980; Teal et al. 1986). While the 2-component blend is considered by some to fully represent the *H. virescens* sex pheromone (Hillier and Baker 2016), and it is formulated commercially for monitoring and surveillance applications, it is clear that several of the other gland compounds, including *Z*11–16:OH, also function as pheromone components by increasing male attraction.

Two requirements need to be satisfied to consider a compound as a sex pheromone component: it must be emitted by the signaler (female *H. virescens*) and it must increase the receiver's (male *H. virescens*) response to a pheromone blend. We consistently detected five pheromone gland components in volatile emissions of calling *H. virescens* females: *Z*9–14:Ald, 14:Ald, *Z*11–16:Ald, 16:Ald, and *Z*11–16:OH. Two additional aldehydes, *Z*7–16:Ald and *Z*9–16:Ald, were detected in some, but not all, volatile collections, and could not consistently be resolved on a DB-5 column; but they are clearly emitted by calling females and they could be resolved on polar columns as in Groot et al. (2014). The release rates of each of the four emitted aldehydes was strongly positively correlated with their respective content in the pheromone gland, consistent with previous findings from both volatile collections (Pope et al. 1982; Heath et al. 1991) and from surface rubs of the pheromone gland with PDMS fibers (Lievers and Groot 2016). Although lab findings have been inconsistent, all six 14- and 16-carbon aldehydes should be considered sex pheromone components of *H. virescens* females (see INTRODUCTION).

*Z*11–16:OH is particularly abundant in the pheromone gland of *H. virescens* (Klun et al. 1979; Klun et al. 1980; Pope et al. 1982; Teal et al. 1986; Heath et al. 1991; Groot et al. 2009; Groot et al. 2010; Groot et al. 2014; Lievers and Groot 2016). Yet previous studies could not detect *Z*11–16:OH in pheromone gland emissions (Pope et al. 1982; Teal et al. 1986; Heath et al. 1991), and even recent surface rubs of the pheromone gland with PDMS fibers did not detect this alcohol (Lievers and Groot 2016). Although we used only four females, we were able to detect and quantify *Z*11–16:OH emissions from all four calling females. Critical to our strategy were (a) the use of a thick-film fused silica megabore column as a collection trap for volatiles, which enabled high trapping efficiency and minimal solvent use for eluting the analytes; (b) silanization of all active surfaces, especially glass surfaces to which *Z*11–16:OH could adsorb; (c) optimization of flow rates to maximize trapping efficiency while minimizing disturbance to the calling female; and (d) derivatization of *Z*11–16:OH to increase its volatility, decrease reactivity, and increase sensitivity of detection by FID and MS.

Unlike the aldehydes, however, we found a negative correlation between the amount of *Z*11–16:OH in the pheromone gland and its release rate in the emitted blend. This observation suggests a dual role for *Z*11–16:OH: as precursor to the major pheromone component *Z*11–16:Ald (Choi et al. 2005; Jurenka et al. 2016) through the action of alcohol oxidases within the pheromone gland or on its cuticular surface (Teal and Tumlinson 1986, 1988), and as a behaviorally active component of the emitted sex pheromone blend. Notably, all previous flight tunnel assays could not attribute any conspecific behavioral function to *Z*11–16:OH (Vetter and Baker 1983; Teal et al. 1986), whereas trapping assays in the field have consistently documented that its addition to either a 2-component blend or a 6-component blend significantly increased trap catch (Hartstack et al. 1980; Klun et al. 1980; Ramaswamy et al. 1985; Shaver et al. 1989).

We confirmed in field studies a clear behavioral function for emitted *Z*11–16:OH. The addition of 1% *Z*11–16:OH to various pheromone blends significantly increased trap catch of *H. virescens* males in most of the experiments that we conducted. This observation was consistent when *Z*11–16:OH was added to a 2-component minimal blend, a 4-component blend, as well as a 6-component aldehyde blend. Moreover, these results were consistent with two different septum loadings (relative to 100 and 300 μg *Z*11–16:Ald) that would result in different release rates, and in experiments conducted in North Carolina and Texas, suggesting no major differences in the use of *Z*11–16:OH across populations. Finally, behavioral observations in the field in 2014 confirmed the 2013 trapping experiment. The addition of 1% *Z*11–16:OH to a 4-component aldehyde blend significantly increased the number of males that were attracted to pheromone-loaded septa, approached them, and contacted them.

While low amounts of *Z*11–16:OH increased trap catch in field assays, higher blend ratios (≥ 5%) of *Z*11–16:OH significantly reduced trap catch (Ramaswamy et al. 1985; Shaver et al. 1989). Our trapping experiment in 2013 and behavioral observations in 2014 strongly confirmed this pattern. The addition of 5 and 10% *Z*11–16:OH, relative to *Z*11–16:Ald, significantly suppressed *H. virescens* male attraction, approach, contact with the septum, and ultimately trap catch. Notably, *H. subflexa* females, a sympatric congener with whom *H. virescens* can hybridize, emit larger amounts of *Z*11–16:OH as a critical component of their sex pheromone blend (Teal et al. 1981; Heath et al. 1990; Heath et al. 1991; Groot et al. 2007; Nojima et al. 2018). In *H. subflexa*, omission of the alcohol, as well as high doses of it, reduced trap catch, whereas intermediate doses increased trap catch; the percentage of *Z*11–16:OH that is attractive to *H. subflexa* is broader than in *H. virescens* (Groot et al. 2007). These results suggest that *H. virescens*

females have tuned the release rate of *Z*11–16:OH to low levels that facilitate the attraction of conspecific males, and male *H. virescens* are attracted to low emissions of *Z*11–16:OH in the proper aldehyde blend and repelled by high emissions of *Z*11–16:OH that could result in interspecific mating.

While we are confident that calling females naturally emit *Z*11–16:OH and that males are more attracted to sex pheromone blends that contain *Z*11–16:OH, we remain cautious about quantifying the rate of emission of this alcohol relative to the major component, *Z*11–16:Ald, because these assays involved only four females. The *Z*11–16:OH content in *H. virescens* pheromone glands was 18.7 ± 11.0% of the *Z*11–16:Ald content. The emission rate of *Z*11–16:OH, however, was only 0.36 ± 0.11% of the *Z*11–16:Ald emission. Heath et al. (1990) determined that septa loaded with 5.81% *Z*11–16:OH relative to *Z*11–16:Ald emitted 2.60% *Z*11–16:OH relative to *Z*11–16:Ald. Extrapolating from these results, our septa, which were loaded with 1% *Z*11–16:OH relative to *Z*11–16:Ald, would emit 0.45% *Z*11–16:OH relative to *Z*11–16:Ald, similar to the 0.36% emitted by females. We note, however, that (a) emission rates from freely calling females in an "open" system would be affected by the air flow over the female; (b) our collection apparatus was not fully optimized for recovering alcohols; and (c) the extrapolations from Heath et al. (1990) are from septa loaded with a 7-component *H. subflexa* blend that included 16-carbon acetate esters.

We conclude that *Z*11–16:OH should be considered a pheromone component of *H. virescens*, with significant effects on conspecific male attraction and trap catch. This compound appears to be under selection pressure in *H. virescens* to be emitted in a specific ratio to other components so as to maximize attraction of conspecific males and avoid mating with closely related sympatric species.

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